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**CELLULASE FUSION PROTEIN
AND
HETEROLOGOUS CELLULASE FUSION CONSTRUCT
ENCODING THE SAME**

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SPONSORED RESEARCH AND DEVELOPMENT

15 Portions of this work were funded by Subcontract No. ZCO-0-30017-01 with the National Renewable Energy Laboratory under Prime Contract No. DE-AC36-99G010337 with the United States Department of Energy. Accordingly the United States Government may have certain rights in this invention.

RELATED APPLICATIONS

20 The present application claims priority to U.S. Provisional Patent Application Serial No. 60/556,711, entitled "Cellulase Fusion Protein and Heterologous Fusion Construct Encoding the Same" and filed on March 25, 2004.

FIELD OF THE INVENTION

25 The present invention relates to a heterologous cellulase fusion construct, which encodes a fusion protein having cellulolytic activity comprising a first catalytic domain derived from a fungal exo-cellobiohydrolase and a second catalytic domain derived from a cellulase enzyme. The invention also relates to vectors and fungal host cells comprising the heterologous cellulase fusion construct as well as methods
30 for producing said cellulase fusion protein and enzymatic cellulase compositions.

BACKGROUND OF THE INVENTION

35 Cellulose and hemicellulose are the most abundant plant materials produced by photosynthesis. They can be degraded and used as an energy source by numerous microorganisms, including bacteria, yeast and fungi, which produce extracellular enzymes capable of hydrolysis of the polymeric substrates to monomeric sugars (Aro *et al.*, 2001). As the limits of non-renewable resources approach, the potential of cellulose

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to become a major renewable energy resource is enormous (Krishna *et al.*, 2001). The effective utilization of cellulose through biological processes is one approach to overcoming the shortage of foods, feeds, and fuels (Ohmiya *et al.*, 1997).

Cellulases are enzymes that hydrolyze cellulose (beta-1,4-glucan or beta D-
5 glucosidic linkages) resulting in the formation of glucose, cellobiose, celooligosaccharides, and the like. Cellulases have been traditionally divided into three major classes: endoglucanases (EC 3.2.1.4) ("EG"), exoglucanases or cellobiohydrolases (EC 3.2.1.91) ("CBH") and beta-glucosidases ([beta]-D-glucoside glucohydrolase; EC 3.2.1.21) ("BG") (Knowles *et al.*, 1987 and Schulein, 1988).
10 Endoglucanases act mainly on the amorphous parts of the cellulose fiber, whereas cellobiohydrolases are also able to degrade crystalline cellulose.

Cellulases are known to be produced by a large number of bacteria, yeast and fungi. Certain fungi produce a complete cellulase system capable of degrading crystalline forms of cellulose, such that the cellulases are readily produced in large
15 quantities via fermentation.

In order to efficiently convert crystalline cellulose to glucose the complete cellulase system comprising components from each of the CBH, EG and BG classifications is required, with isolated components less effective in hydrolyzing crystalline cellulose (Filho *et al.*, 1996). In particular, the combination of EG-type
20 cellulases and CBH- type cellulases interact to more efficiently degrade cellulose than either enzyme used alone (Wood, 1985; Baker *et al.*, 1994; and Nieves *et al.*, 1995).

Additionally, cellulases are known in the art to be useful in the treatment of textiles for the purposes of enhancing the cleaning ability of detergent compositions, for use as a softening agent, for improving the feel and appearance of cotton fabrics, and
25 the like (Kumar *et al.*, 1997). Cellulase-containing detergent compositions with improved cleaning performance (US Pat. No. 4,435,307; GB App. Nos. 2,095,275 and 2,094,826) and for use in the treatment of fabric to improve the feel and appearance of the textile (US Pat. Nos. 5,648,263, 5,691,178, and 5,776,757, and GB App. No. 1,358,599), have been described in the literature.

30 Hence, cellulases produced in fungi and bacteria have received significant attention. In particular, fermentation of *Trichoderma* spp. (e.g., *Trichoderma longibrachiatum* or *Trichoderma reesei*) has been shown to produce a complete cellulase system capable of degrading crystalline forms of cellulose. Over the years, *Trichoderma* cellulase production has been improved by classical mutagenesis,
35 screening, selection and development of highly refined, large scale inexpensive

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fermentation conditions. While the multi-component cellulase system of *Trichoderma spp.* is able to hydrolyze cellulose to glucose, there are cellulases from other microorganisms, particularly bacterial strains, with different properties for efficient cellulose hydrolysis, and it would be advantageous to express these proteins in a filamentous fungus for industrial scale cellulase production. However, the results of many studies demonstrate that the yield of bacterial enzymes from filamentous fungi is low (Jeeves *et al.*, 1991).

In this invention, a heterologous cellulase fusion construct, which includes the coding region of a fungal exo-cellobiohydrolase (CBH) catalytic domain fused to a coding region of a cellulase catalytic domain, has been introduced and expressed in a filamentous fungi host cell to increase the yield and effectiveness of cellulase enzymes.

SUMMARY OF THE INVENTION

In a first aspect, the invention includes a heterologous cellulase fusion construct comprising in operable linkage from the 5' end of said construct, (a) a DNA molecule encoding a signal sequence, (b) a DNA molecule encoding a first catalytic domain, wherein said first catalytic domain is derived from of a fungal exo-cellobiohydrolase, and (c) a DNA molecule encoding a second catalytic domain, wherein said second catalytic domain is the catalytic domain of a cellulase enzyme.

In a first embodiment of this aspect, the heterologous cellulase fusion construct further comprises a linker sequence located 3' of the first catalytic domain and 5' of the second catalytic domain. In a second embodiment, the heterologous cellulase fusion construct lacks the cellulose binding domain (CBD) of the exo-cellobiohydrolase. In a third embodiment, the heterologous cellulase fusion construct further comprises a kexin site located after the linker sequence and before the second catalytic domain. In a fourth embodiment, the heterologous fusion construct will comprise a promoter of a filamentous fungus secretable protein, said promoter located in operable linkage 5' of the first catalytic domain. In a fifth embodiment, the promoter is a *cbh* promoter and preferably a *cbh1* promoter derived from *T. reesei*. In a sixth embodiment, the first catalytic domain is derived from a CBH1 exo-cellobiohydrolase and particularly a CBH1 having an amino acid sequence of at least 90% sequence identity with the sequence set forth in SEQ ID NO.: 6. In a seventh embodiment, the second catalytic domain is an endoglucanase catalytic domain. In an eighth embodiment, the second catalytic domain is an exo-cellobiohydrolase catalytic domain. In a ninth embodiment, the second catalytic domain is derived from a bacterial cellulase. In a tenth embodiment, the second

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catalytic domain is selected from the group consisting of an *Acidothermus cellulolyticus* GH5A endoglucanase I (E1) catalytic domain; an *Acidothermus cellulolyticus* GH48 (GH48) cellulase catalytic domain; an *Acidothermus cellulolyticus* GH74 endoglucanase (GH74-EG) catalytic domain; a *Thermobifida fusca* E3 (Tf-E3) cellulase catalytic domain; and a *Thermobifida fusca* E5 endoglucanase (Tf-E5) catalytic domain. In an eleventh embodiment, the heterologous cellulase fusion construct lacks the cellulose binding domain of the exo-cellbiohydrolase of the first catalytic domain and the cellulose binding

domain of the cellulase of the second catalytic domain. In a twelfth embodiment, the second catalytic domain is an *Acidothermus cellulolyticus* GH5A E1 catalytic domain and particularly the *Acidothermus cellulolyticus* GH5A E1 catalytic domain having an amino acid sequence of at least 90% sequence identity with the sequence set forth in SEQ ID NO. 8. In a thirteenth embodiment, the heterologous cellulase fusion construct comprises a terminator sequence located 3' to the second catalytic domain. In a fourteenth embodiment, the heterologous fusion construct comprises a selectable marker.

In a second aspect, the invention includes a vector comprising in operable linkage from the 5' end, a promoter of a filamentous fungus secretable protein, a DNA molecule encoding a signal sequence, a DNA molecule encoding a first catalytic domain, wherein said first catalytic domain is derived from a fungal exo-cellobiohydrolase, a DNA molecule encoding a second catalytic domain, wherein said second catalytic domain is the catalytic domain of a cellulase and a terminator. In one embodiment, the vector will further include a selectable marker. In a second embodiment, the vector will comprise a linker located 3' of the first catalytic domain and 5' of the second catalytic domain. In a third embodiment, the vector will lack the DNA sequence encoding cellulose binding domain of the first catalytic domain. In a fourth embodiment, the vector will comprise a kexin site. In a fifth embodiment, the second catalytic domain is derived from a bacterial cellulase. In a sixth embodiment, the vector lacks the DNA sequence encoding cellulose binding domain of the cellulase of the second catalytic domain.

In a third aspect, the invention includes a fungal host cell transformed with a heterologous cellulase fusion construct or a fungal host cell transformed with a vector comprising a heterologous cellulase fusion construct as described herein.

In a fourth aspect, the invention includes a recombinant fungal cell comprising the heterologous cellulase fusion construct or a vector comprising the same.

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In a particularly preferred embodiment of the third and fourth aspects, the fungal host cell is a *Trichoderma* host cell and more particularly a strain of *T. reesei*. In another embodiment of these aspects, native cellulase genes, such as *cbh1*, *cbh2*, *egl1* and *egl2* have been deleted from the fungal cells. In a third embodiment, the native
5 cellulose binding domain has been deleted from the fungal cells.

In a fifth aspect, the invention includes an isolated cellulase fusion protein having cellulolytic activity which comprises a first catalytic domain, wherein said first catalytic domain is an exo-cellobiohydrolase catalytic domain and a second catalytic domain, wherein said second catalytic domain is derived from a cellulase. In one
10 embodiment of this aspect, the exo-cellobiohydrolase is a CBH1. In a second embodiment, the second catalytic domain is derived from a bacterial cellulase. In a third embodiment, the bacterial cellulase is an endoglucanase and in another embodiment the bacterial cellulase is an exo-cellobiohydrolase. In a fourth embodiment, the bacterial cellulase is derived from a strain of *Acidothermus cellulolyticus*. In a fifth embodiment,
15 the invention concerns a cellulolytic composition comprising the isolated cellulase fusion protein.

In a sixth aspect, the invention includes a method of producing an enzyme having cellulolytic activity comprising, a) stably transforming a filamentous fungal host cell with a heterologous cellulase fusion construct or vector as defined above in the first
20 aspect and second aspect; b) cultivating the transformed fungal host cell under conditions suitable for said fungal host cell to produce an enzyme having cellulolytic activity; and c) recovering said enzyme.

In one embodiment of this aspect, the filamentous fungal host cell is a *Trichoderma* cell, and particularly a *T. reesei* host cell. In a second embodiment, the
25 exo-cellobiohydrolase is a CBH1 and the cellulase is an *Acidothermus cellulolyticus* cellulase or a *Thermobifida fusca* cellulase. In a third embodiment, the recovered enzyme is a cellulase fusion protein, components of the cellulase fusion protein, or a combination of the cellulase fusion protein and the components thereof. In a fourth embodiment, the recovered enzyme(s) is purified.

In an seventh aspect, the invention includes a *Trichoderma* host cell which expresses a cellulase fusion protein, wherein said fusion protein comprises a first catalytic domain, wherein the catalytic domain is derived from an exo-cellobiohydrolase and a second catalytic domain, wherein the second catalytic domain is derived from a cellulase enzyme. In one embodiment, the *Trichoderma* host cell is a *T. reesei* cell. In a
30 second embodiment, the exo-cellobiohydrolase is a CBH1 and the cellulase is a
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bacterial cellulase. In a third embodiment, the bacterial cellulase is derived from an *Acidothermus cellulolyticus* cellulase and particularly an *Acidothermus cellulolyticus* E1, GH48 or GH74 cellulase. In a fourth embodiment, the fusion protein will lack the CBD of the cellulase and in other embodiments the fusion protein will include the CBD of the cellulase. In a fifth embodiment, the *T. reesei* host cell includes deleted native cellulase genes.

In an eighth aspect, the invention includes a fungal cellulase composition comprising a cellulase fusion protein or components thereof, wherein the fusion protein or components thereof is the product of a recombinant *Trichoderma* spp.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a representation of a heterologous cellulase fusion construct encompassed by the invention, which includes a *Trichoderma reesei cbh1* promoter, a *cbh1* core (*cbh1* signal sequence and *cbh1* catalytic domain), a *cbh1* linker sequence, a kexin site, an E1 core (an *Acidothermus cellulolyticus* E1 endoglucanase catalytic domain), a *cbh1* terminator and an *A. nidulans amdS* selectable marker.

Figure 2 is a DNA sequence (SEQ ID NO: 1) of the *T. reesei cbh1* signal sequence (SEQ ID NO: 2); the *T. reesei cbh1* catalytic domain (SEQ ID NO: 3), and the *T. reesei cbh1* linker (SEQ ID NO: 4). The signal sequence is underlined, the catalytic domain is in bold, and the linker sequence is in italics.

Figure 3 shows the predicted amino acid sequence (SEQ ID NO: 5) based on the nucleotide sequence provided in Figure 2, wherein the signal peptide is underlined, the catalytic domain, represented by (SEQ ID NO: 6), is in bold, and the linker is in italics.

Figure 4 is an illustration of a nucleotide sequence (SEQ ID NO: 7) encoding an *Acidothermus cellulolyticus* GH5A endoglucanase I (E1) catalytic domain.

Figure 5 is the predicted amino acid sequence (SEQ ID NO: 8) of the *Acidothermus cellulolyticus* GH5A E1 catalytic domain based on the nucleotide sequence provided in Figure 4.

Figure 6 is an illustration of a nucleotide sequence (SEQ ID NO: 9) encoding an *Acidothermus cellulolyticus* GH48 cellulase catalytic domain.

Figure 7 is the predicted amino acid sequence (SEQ ID NO: 10) of the *Acidothermus cellulolyticus* GH48 catalytic domain based on the nucleotide sequence provided in Figure 6.

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Figures 8A-8B are an illustration of a nucleotide sequence (SEQ ID NO: 11) encoding an *Acidothermus cellulolyticus* GH74- endoglucanase (EG) catalytic domain.

Figure 9 is the predicted amino acid sequence (SEQ ID NO: 12) of the *Acidothermus cellulolyticus* GH74-EG based on the nucleotide sequence provided in
5 Figures 8A and 8B.

Figure 10 is an illustration of a nucleotide sequence (SEQ ID NO: 13) encoding the CBD, linker and catalytic domain of a *Thermobifida fusca* E-3 (TfE-3) cellulase.

Figure 11 is the predicted amino acid sequence (SEQ ID NO: 14) of the TfE-3 CBD, linker and catalytic domain based on the nucleotide sequence provided in Figure
10 10.

Figure 12 is an illustration of a nucleotide sequence (SEQ ID NO: 15) encoding the CBD, linker and catalytic domain of a *Thermobifida fusca* endoglucanase 5 (TfE5).

Figure 13 is the predicted amino acid sequence (SEQ ID NO: 16) of the TfE5 CBD, linker and catalytic domain based on the nucleotide sequence provided in Figure
15 12.

Figure 14 is the nucleotide sequence(2656 bases) (SEQ ID NO: 17) of a heterologous cellulase fusion construct described in example 1 comprising, the *T. reesei* CBH1 signal sequence; the catalytic domain of the *T. reesei* CBH1; the *T. reesei* CBH1 linker sequence; a kexin cleavage site which includes codons for the amino
20 acids, SKR, and the sequence coding for the *Acidothermus cellulolyticus* GH5A-E1 catalytic domain.

Figure 15 is the predicted amino acid sequence (SEQ ID NO: 18) of the cellulase fusion protein based on the nucleic acid sequence in Figure 14.

Figure 16 provides a schematic diagram of the pTrex4 plasmid, which was used
25 for expression of a heterologous fusion cellulase construct (CBH1-endoglucanase) as described in the examples and includes the *Trichoderma reesei cbh1* promoter, the *T. reesei* CBH1 signal sequence, catalytic domain, and linker sequences, a kexin cleavage site and a cellulase gene of interest (in this example an endoglucanase) inserted between a SpeI and Ascl site, a *cbh1 Trichoderma reesei* terminator and the
30 *amdS Aspergillus nidulans* acetamidase marker gene.

Figures 17A - E provide the nucleotide sequence (SEQ ID NO:19) (10239 bp) of the pTrex4 plasmid of Figure 16 excluding the cellulase gene of interest which includes the cellulose catalytic domain.

Figure 18 illustrates a SDS-PAGE gel of supernate samples of shake flask
35 growth of clones of a *T. reesei* strain deleted for the cellulases, *cbh1*, *cbh2*, *egl1* and

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egl2 and transformed with the CBH1-E1 fusion construct. Lanes 1 and 10 represent MARK 12 Protein Standard (Invitrogen, Carlsbad, CA). Lanes 2 – 8 represent various transformants and lane 9 represents the untransformed *T. reesei* strain. The upper arrow indicates the cellulase fusion protein and the lower arrow indicates the cleaved E1 catalytic domain.

Figure 19 illustrates a SDS-PAGE gel of supernate samples of shake flask growth of clones of a *T. reesei* strain deleted for the cellulases, *cbh1*, *cbh2*, *egl1* and *egl2* and transformed with the CBH1-GH48 fusion construct. Lane 1 represents the untransformed control. Lane 2 represents MARK12 Protein Standard (Invitrogen, Carlsbad, CA). Lanes 3 – 12 represent various transformants. The arrow indicates the CBH1-GH48 fusion protein.

Figure 20 illustrates a SDS-PAGE gel of supernate samples of shake flask growth of clones of a *T. reesei* strain deleted for the cellulases, *cbh1*, *cbh2*, *egl1* and *egl2* and transformed with the CBH1-GH74 fusion construct. Lane 1 represents the untransformed control. Lane 3 represents MARK 12 Protein Standard (Invitrogen, Carlsbad, CA). Lanes 2 and 4 – 12 represent various transformants. The upper arrow around indicates the CBH1-GH74 fusion protein and the lower arrow indicates the cleaved GH74 catalytic domain.

Figure 21 illustrates a SDS-PAGE gel of supernate samples of shake flask growth of clones of a *T. reesei* strain deleted for the cellulases, *cbh1*, *cbh2*, *egl1* and *egl2* and transformed with the CBH1-TfE3 fusion construct. Lane 1 represents MARK 12 Protein Standard (Invitrogen, Carlsbad, CA). Lanes 2 – 12 represent various transformants. The arrow indicates new bands observed in the CBH1-TfE-3 fusion expressing transformants.

Figure 22 illustrates a SDS-PAGE gel of supernate samples of shake flask growth of clones of a *T. reesei* strain deleted for the cellulases, *cbh1*, *cbh2*, *egl1* and *egl2* and transformed with the CBH1-TfE5 fusion construct. Lane 1 represents MARK 12 Protein Standard (Invitrogen). Lane 2 represents the untransformed strain and lanes 3 – 12 represent various transformants. Arrows indicate new bands observed in the CBH1-TfE5 fusion expressing transformants.

Figure 23 illustrates the % cellulose conversion to soluble sugars over time for a *T. reesei* parent strain comprising native cellulase genes with a corresponding *T. reesei* strain which expresses the CBH1-E1 fusion protein and reference is made to example 3.

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DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Practitioners are particularly directed to Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (Second and Third Editions), Cold Spring Harbor Press, Plainview, N.Y., 1989 and 2001, and Ausubel FM *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1993, for definitions and terms of the art.

It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the scope and spirit of the invention will become apparent to one skilled in the art from this detailed description.

1. DEFINITIONS

The term "heterologous cellulase fusion construct" refers to a nucleic acid construct that is composed of parts of different genes in operable linkage. The components include from the 5' end a DNA molecule encoding a first catalytic domain, wherein said first catalytic domain is a fungal exo-cellobiohydrolase catalytic domain

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and a DNA molecule encoding a second catalytic domain, wherein said second catalytic domain is a cellulase catalytic domain.

The term "cellulase fusion protein" or "fusion protein having cellulolytic activity" refers to an enzyme which exhibits cellulolytic activity and which has both a fungal exo-cellobiohydrolase catalytic domain and a cellulase catalytic domain.

The term "components of a cellulase fusion protein" refers to individual (cleaved) fragments of the cellulase fusion protein, wherein each fragment has cellulolytic activity and includes either the first or the second catalytic domain of the fusion protein.

The term "cellulase" refers to a category of enzymes capable of hydrolyzing cellulose (beta-1,4-glucan or beta D-glucosidic linkages) polymers to shorter cello-oligosaccharide oligomers, cellobiose and/or glucose.

The term "exo-cellobiohydrolase" (CBH) refers to a group of cellulase enzyme classified as EC 3.2.1.91. These enzymes are also known as exoglucanases or cellobiohydrolases. CBH enzymes hydrolyze cellobiose from the reducing or non-reducing end of cellulose. In general a CBH1 type enzyme preferentially hydrolyzes cellobiose from the reducing end of cellulose and a CBH2 type enzyme preferentially hydrolyzes the non-reducing end of cellulose.

The term "endoglucanase" (EG) refers to a group of cellulase enzymes classified as EC 3.2.1.4. An EG enzyme hydrolyzes internal beta-1,4 glucosidic bonds of the cellulose.

The term "beta-glucosidases" refers to a group of cellulase enzymes classified as EC 3.2.1.21.

"Cellulolytic activity" encompasses exoglucanase activity, endoglucanase activity or both types of enzymatic activity.

The term "catalytic domain" refers to a structural portion or region of the amino acid sequence of a cellulase which possess the catalytic activity of the cellulase. The catalytic domain is a structural element of the cellulase tertiary structure that is distinct from the cellulose binding site, which is a structural element which binds the cellulase to a substrate such as cellulose.

The term "cellulose binding domain (CBD)" as used herein refers to a portion of the amino acid sequence of a cellulase or a region of the enzyme that is involved in the cellulose binding activity of a cellulase. Cellulose binding domains generally function by non-covalently binding the cellulase to cellulose, a cellulose derivative or other

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polysaccharide equivalent thereof. CBDs typically function independent of the catalytic domain.

The term "first catalytic domain" refers to the catalytic domain of a fungal exo-cellobiohydrolase.

5 The term "second catalytic domain" or "cellulase catalytic domain" refers to the catalytic domain of a cellulase enzyme, wherein the cellulase enzyme may be an exo-cellobiohydrolase or an endoglucanase and wherein the catalytic domain is operably linked to the 3' end of the first catalytic domain.

10 A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA encoding a signal peptide is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it affects the transcription of the sequence. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of
15 the heterologous cellulase fusion construct contiguous and in reading frame.

 As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, that may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between
20 individual coding segments (exons).

 The term "polypeptide" as used herein refers to a compound made up of a single chain of amino acid residues linked by peptide bonds. The term "protein" as used herein may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides.

25 The term "nucleic acid molecule", "nucleic acid" or "polynucleotide" includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein such as a cellulase fusion protein, may be produced.

 A "heterologous" nucleic acid sequence has a portion of the sequence, which is
30 not native to the cell in which it is expressed. For example, heterologous, with respect to a control sequence refers to a control sequence (*i.e.* promoter or enhancer) that does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid sequences are not endogenous to the cell or part of the genome in which they are present, and have been added to the cell,
35 by infection, transfection, transformation, microinjection, electroporation, or the like. A

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"heterologous" nucleic acid sequence may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native cell. The term heterologous nucleic acid sequence encompasses a heterologous cellulase fusion construct according to the invention.

As used herein, the term "vector" refers to a nucleic acid sequence or construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA sequences in a foreign cell. An expression vector may be generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, virus, or nucleic acid fragment.

• As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

As used herein, the term "selectable marker" refers to a nucleotide sequence which is capable of expression in cells and where expression of the selectable marker confers to cells containing the expressed gene the ability to grow in the presence of a corresponding selective agent, or under corresponding selective growth conditions.

As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a downstream gene. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") are necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

The term "signal sequence" or "signal peptide" refers to a sequence of amino acids at the N-terminal portion of a protein, which facilitates the secretion of the mature form of the protein outside the cell. The mature form of the extracellular protein lacks the signal sequence which is cleaved off during the secretion process.

By the term "host cell" is meant a cell that contains a heterologous cellulase fusion construct encompassed by the invention or a vector including the same and supports the replication, and/or transcription or transcription and translation (expression) of the heterologous cellulase fusion construct. Host cells for use in the

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present invention can be prokaryotic cells, such as *E. coli*, or eukaryotic cells such as yeast, plant, insect, amphibian, or mammalian cells. In general, host cells are filamentous fungi.

The term "filamentous fungi" includes all filamentous fungi recognized by those of skill in the art. A preferred fungus is selected from the subdivision Eumycota and Oomycota and particularly from the group consisting of *Aspergillus*, *Trichoderma*, *Fusarium*, *Chrysosporium*, *Penicillium*, *Humicola*, *Neurospora*, or alternative sexual forms thereof such as *Emericella* and *Hypocrea* (See, Kuhls et al., 1996).

The filamentous fungi are characterized by vegetative mycelium having a cell wall composed of chitin, glucan, chitosan, mannan, and other complex polysaccharides, with vegetative growth by hyphal elongation and carbon catabolism that is obligately aerobic.

The term "derived" encompasses the terms, originated from, obtained or obtainable from, and isolated from.

An "equivalent" amino acid sequence is an amino acid sequence that is not identical to an original reference amino acid sequence but includes some amino acid changes, which may be substitutions, deletions, additions or the like, wherein the protein exhibits essentially the same qualitative biological activity of the reference protein. An equivalent amino acid sequence will have between 80% - 99% amino acid identity to the original reference sequence. Preferably the equivalent amino acid sequence will have at least 85%, 90%, 93%, 95%, 96%, 98% and 99% identity to the reference sequence.

A "substitution" results from the replacement of one or more nucleotides or amino acid by different nucleotides or amino acids, respectively. Substitutions are usually made in accordance with known conservative substitutions, wherein one class of amino acid is substituted with an amino acid in the same class. A "non-conservative substitution" refers to the substitution of an amino acid in one class with an amino acid from another class.

A "deletion" is a change in a nucleotide or amino acid sequence in which one or more nucleotides or amino acids are absent.

An "addition" is a change in a nucleotide or amino acid sequence that has resulted from the insertion of one or more nucleotides or amino acid as compared to an original reference sequence.

As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of heterologous nucleic acid sequences or that the

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cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

5 As used herein, the terms "transformed", "stably transformed" or "transgenic" with reference to a cell means the cell has a heterologous nucleic acid sequence according to the invention integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

10 The term "introduced" in the context of inserting a heterologous cellulase fusion construct or heterologous nucleic acid sequence into a cell, means "transfection", "transformation" or "transduction" and includes reference to the incorporation of a heterologous nucleic acid sequence or heterologous cellulase fusion construct into a eukaryotic or prokaryotic cell where the heterologous nucleic acid sequence or heterologous cellulase nucleic acid construct may be incorporated into the genome of
15 the cell (for example, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process
20 includes both transcription and translation.

It follows that the term "cellulase fusion protein expression" or "fusion expression" refers to transcription and translation of a "heterologous cellulase fusion construct" comprising a first catalytic domain fused to a second cellulase catalytic domain, the products of which include precursor RNA, mRNA, polypeptide, post-
25 translationally processed polypeptides, and derivatives thereof.

As used herein, the term "purifying" generally refers to subjecting recombinant nucleic acid or protein containing cells to biochemical purification and/or column chromatography.

30 As used herein, the terms "active" and "biologically active" refer to a biological activity associated with a particular protein, such as the enzymatic activity associated with a cellulase. It follows that the biological activity of a given protein refers to any biological activity typically attributed to that protein by those of skill in the art.

As used herein, the term "enriched" means that the concentration of a cellulase enzyme found in a fungal cellulase composition is greater relative to the concentration

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found in a wild type or naturally occurring fungal cellulase composition. The terms enriched, elevated and enhanced may be used interchangeably herein.

A "wild type fungal cellulase composition" is one produced by a naturally occurring fungal source and which comprises one or more BG, CBH and EG components wherein each of these components is found at the ratio produced by the fungal source.

Thus, to illustrate, a naturally occurring cellulase system may be purified into substantially pure components by recognized separation techniques well published in the literature, including ion exchange chromatography at a suitable pH, affinity chromatography, size exclusion and the like. A purified cellulase fusion protein or components thereof may then be added to the enzymatic solution resulting in an enriched cellulase solution. It is also possible to elevate the amount of EG or CBH produced by a microbe by expressing a cellulase fusion protein encompassed by the invention.

"A", "an" and "the" include plural references unless the context clearly dictates otherwise.

As used herein the term "comprising" and its cognates are used in their inclusive sense: that is equivalent to the term "including" and its corresponding cognates.

"ATCC" refers to American Type Culture Collection located in Manassas VA 20108 (ATCC, [www/atcc.org](http://www.atcc.org)).

"NRRL" refers to the Agricultural Research Service Culture Collection, National Center for Agricultural utilization Research (and previously known as USDA Northern Regional Research Laboratory), Peoria, ILL.

2. PREFERRED EMBODIMENTS

A. Components and Construction of Heterologous Cellulase Fusion Constructs and Expression Vectors.

A heterologous cellulase fusion construct or a vector comprising the heterologous cellulase fusion construct may be introduced into and replicated in a filamentous fungal host cell for protein expression and secretion.

The components of the heterologous cellulase fusion construct comprise in operable linkage from the 5' end of said construct, optionally a signal peptide, a DNA molecule encoding a first catalytic domain, wherein said first catalytic domain is derived from a fungal exo-cellobiohydrolase (CBH) and a DNA molecule encoding a second catalytic domain, wherein the second catalytic domain is derived from a cellulase

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enzyme.

In another embodiment, the heterologous cellulase fusion comprises in operable linkage from the 5' end of said construct, a signal peptide, a DNA molecule encoding a CBH catalytic domain, a DNA molecule encoding the CBD of a second cellulase catalytic domain, and a DNA molecule encoding the second cellulase catalytic domain, wherein said cellulase second catalytic domain is derived from a bacterial cellulase.

In another embodiment, the construct will comprise in operable linkage from the 5' end of said construct, a signal peptide, a DNA molecule encoding a catalytic domain of a CBH, optionally a DNA molecule encoding the CBD of the CBH, a linker, optionally a DNA molecule encoding a CBD of a second catalytic domain, and a DNA molecule encoding the second catalytic domain.

In a further embodiment, the heterologous cellulase fusion construct or an expression vector comprises in operable linkage from the 5' end a promoter of a filamentous fungus secretable protein; a DNA molecule encoding a signal sequence; a DNA molecule encoding a first catalytic domain, a linker, optionally a DNA encoding the CBD of a second catalytic domain, a DNA encoding the second catalytic domain, and a terminator.

One preferred expression vector will include in operable linkage from the 5' end, a promoter of a filamentous fungus secretable protein, a DNA molecule encoding a fungal exo-cellobiohydrolase signal sequence, a DNA molecule encoding a catalytic domain of an exo-cellobiohydrolase, a linker, a DNA molecule encoding a cellulase catalytic domain, and a terminator.

In some embodiments the vector will include the CBD of the exo-cellobiohydrolase and in some embodiments the vector will include the CBD of the cellulase of the second catalytic domain. In a preferred embodiment, the coding sequence for the cellulase catalytic domain (either including the cellulase CBD or lacking the cellulase CBD) will not include the cellulase signal sequence. Reference is made to Figures 1, 14 and 16 as examples of embodiments including an expression vector and heterologous cellulase fusion construct of the invention.

Exemplary promoters include both constitutive promoters and inducible promoters. Examples include the promoters from the *Aspergillus niger*, *A. awamori* or *A. oryzae* glucoamylase, alpha-amylase, or alpha-glucosidase encoding genes; the *A. nidulans* *gpdA* or *trpC* Genes; the *Neurospora crassa* *cbh1* or *trp1* genes; the *A. niger* or *Rhizomucor miehei* aspartic proteinase encoding genes; the *T. reesei* *cbh1*, *cbh2*, *egl1*, *egl2*, or other cellulase encoding genes; a CMV promoter, an SV40 early

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promoter, an RSV promoter, an EF-1 α promoter, a promoter containing the tet responsive element (TRE) in the tet-on or tet-off system as described (ClonTech and BASF), the beta actin promoter. In some embodiments the promoter is one that is native to the fungal host cell to be transformed.

5 In one preferred embodiment, the promoter is an exo-cellobiohydrolase *cbh1* or *cbh2* promoter and particularly a *cbh1* promoter, such as a *T. reesei cbh1* promoter. The *T. reesei cbh1* promoter is an inducible promoter, and reference is made to GenBank Accession No. D86235.

The DNA sequence encoding an exo-cellobiohydrolase catalytic domain is operably linked to a DNA sequence encoding a signal sequence. The signal sequence is preferably that which is naturally associated with the exo-cellobiohydrolase to be expressed. Preferably the signal sequence is encoded by a *Trichoderma* or *Aspergillus* gene which encodes a CBH. More preferably the signal sequence is encoded by a *Trichoderma* gene which encodes a CBH1. In further embodiments, the promoter and signal sequence of the heterologous cellulase fusion construct are derived from the same source. In some embodiments, the signal sequence is a *Trichoderma cbh1* signal sequence that is operably linked to a *Trichoderma cbh1* promoter. In further
10
15
embodiments, the signal sequence has the amino acid sequence of SEQ ID NO: 2 or a sequence having at least 95% identity thereto.

20 Most exo-cellobiohydrolases (CBHs) and endoglucanases (EGs) have a multidomain structure consisting of a catalytic domain separated from a cellulose binding domain (CBD) by a linker peptide (Suurnakki *et al.*, 2000). The catalytic domain contains the active site whereas the CBD interacts with cellulose by binding the enzyme to it (van Tilbeurgh *et al.*, 1986 and Tomme *et al.*, 1988).

25 Numerous cellulases have been described in the scientific literature, examples of which include: from *Trichoderma reesei*: Shoemaker, S. *et al.*, Bio/Technology, 1:691-696, 1983, which discloses CBH1; Teeri, T. *et al.*, Gene, 51:43-52, 1987, which discloses CBH2; Penttila, M. *et al.*, Gene, 45:253-263, 1986, which discloses EG1; Saloheimo, M. *et al.*, Gene, 63:11-22, 1988, which discloses EG2; Okada, M. *et al.*, Appl. Environ. Microbiol., 64:555-563, 1988, which discloses EG3; Saloheimo, M. *et al.*, Eur. J. Biochem., 249:584-591, 1997, which discloses EG4; and Saloheimo, A. *et al.*, Molecular Microbiology, 13:219-228, 1994, which discloses EG5. Exo-cellobiohydrolases and endoglucanases from species other than *Trichoderma* have also been described e.g., Ooi *et al.*, 1990, which discloses the cDNA sequence coding
30
35 for endoglucanase F1-CMC produced by *Aspergillus aculeatus*; Kawaguchi T *et al.*,

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1996, which discloses the cloning and sequencing of the cDNA encoding beta-glucosidase 1 from *Aspergillus aculeatus*; Sakamoto *et al.*, 1995, which discloses the cDNA sequence encoding the endoglucanase CMCase-1 from *Aspergillus kawachii* IFO 4308; and Saarilahti *et al.*, 1990 which discloses an endoglucanase from *Erwinia carotovora*. The sequences encoding these enzymes may be used in the heterologous cellulase fusion construct of the invention.

In some embodiments the first catalytic domain is derived from a fungal CBH. In other embodiments the CBH is a CBH1 type exo-cellobiohydrolase and in other embodiments the catalytic domain is derived from a CBH2 type exo-cellobiohydrolase. In some embodiments, the CBH1 catalytic domain is derived from a *Trichoderma* spp.

In one embodiment, the first catalytic domain is encoded by a nucleic acid sequence of a *Trichoderma reesei cbh1*. In some embodiments the nucleic acid is the sequence of SEQ ID NO:3 and nucleotide sequences homologous thereto.

In other embodiments, the first catalytic domain will have the amino acid sequence of SEQ ID NO: 6 and equivalent amino acid sequences thereto. Further DNA sequences encoding any equivalents of said amino acid sequences of SEQ ID NO: 6, wherein said equivalents have a similar qualitative biological activity to SEQ ID NO: 6 may be incorporated into the heterologous cellulase fusion construct.

In some embodiments, heterologous cellulase fusion constructs encompassed by the invention will include a linker located 3' to the sequence encoding the exo-cellobiohydrolase catalytic domain and 5' to the sequence encoding the endoglucanase catalytic domain. In some preferred embodiments, the linker is derived from the same source as the catalytic domain of the exo-cellobiohydrolase. Preferably the linker will be derived from a *Trichoderma cbh1* gene. One preferred linker sequence is illustrated in Figure 3. In other embodiments, the heterologous cellulase fusion construct or an expression vector may include two or more linkers. For example, a linker may be located not only between the coding sequence of the first catalytic domain and the coding sequence of the second catalytic domain, but also between the coding region of the CBD of the second catalytic domain and the coding region of the second catalytic domain. In general a linker may be between about 5 to 60 amino acid residues, between about 15 to 50 amino acid residues, and between about 25 to 45 amino acid residues. Reference is made to Srisodsuk M. *et al.*, 1993 for a discussion of the linker peptide of *T. reesei* CBH1.

In addition to the linker sequence, a heterologous cellulase fusion construct or expression vector including a cellulase fusion construct may include a cleavage site,

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such as a protease cleavage site. In one preferred embodiment, the cleavage site is a kexin site which encodes the dipeptide Lys-Arg.

The heterologous cellulase fusion constructs include a coding sequence for a second catalytic domain of a cellulase. The cellulase may be from a fungal or bacterial source. Additionally, the cellulase may be an exo-cellobiohydrolase (CBH) or an endoglucanase (EG). In some preferred embodiments, the second catalytic domain will be derived from a bacterial cellulase. Source of both CBH and EG cellulases are mentioned above. Additionally, endoglucanases are found in more than 13 of the Glycosyl Hydrolase families using the classification of Coutinho, P. M. et al. (1999) Carbohydrate-Active Enzymes (CAZy) server at afmb.cnrs-mrs.fr/~cazy/CAZY/index.

Particularly preferred DNA sequences encoding a second catalytic domain of a bacterial cellulase include:

- a) the DNA of SEQ ID NO: 7 encoding an *Acidothermus cellulolyticus* GH5A endoglucanase I (E1) catalytic domain having amino acid sequence SEQ ID NO: 8;
- 15 b) the DNA sequence of SEQ ID NO: 9 encoding an *Acidothermus cellulolyticus* GH48 cellulase catalytic domain having amino acid sequence SEQ ID NO: 10;
- c) the DNA of SEQ ID NO: 11 encoding an *Acidothermus cellulolyticus* GH74 endoglucanase catalytic domain having amino acid sequence SEQ ID NO: 12;
- d) the DNA of SEQ ID NO: 13 encoding a *Thermobifida fusca* E3 cellulase
20 having amino acid sequence SEQ ID NO: 14;
- e) the DNA sequence of SEQ ID NO: 15 encoding a *Thermobifida fusca* E5 endoglucanase having amino acid sequence SEQ ID NO: 16 and
- f) DNA sequences or homologous DNA sequences encoding any equivalents of said amino acid sequences of SEQ ID NOs: 8, 10, 12, 14 and 16, wherein said
25 equivalents have a similar qualitative biological activity to said sequences.

In some preferred embodiments the endoglucanase is an *Acidothermus cellulolyticus* E1 and reference is made to the an *Acidothermus cellulolyticus* endoglucanases disclosed in WO 9105039; WO 9315186; USP 5,275,944; WO 9602551; USP 5,536,655 and WO 0070031. Also reference is made to GenBank
30 U33212. In some embodiments, the *Acidothermus cellulolyticus* E1 has an amino acid sequence of a least 90%, 93%, 95% and 98% sequence identity with the sequence set forth in SEQ ID NO: 6.

As stated above homologous nucleic acid sequences to the nucleic acid sequences illustrated in SEQ ID NOs: 1, 3, 7, 9, 11, 13, and 15 may be used in a
35 heterologous cellulase fusion construct or vector according to the invention.

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Homologous sequences include sequences found in other species, naturally occurring allelic variants and biologically active functional derivatives. A homologous sequence will have at least 80%, 85%, 88%, 90%, 93%, 95%, 97%, 98% and 99% identity to one of the sequences of SEQ ID NOs: 1, 3, 7, 9, 11, 13 and 15 when aligned using a
5 sequence alignment program. For example, a homologue of a given sequence has greater than 80% sequence identity over a length of the given sequence e.g., the coding sequence for the Tf-E3 catalytic domain as described herein.

For a given heterologous cellulase fusion construct or components of the construct it is appreciated that as a result of the degeneracy of the genetic code, a
10 number of coding sequences can be produced that encode a protein having the same amino acid sequence. For example, the triplet CGT encodes the amino acid arginine. Arginine is alternatively encoded by CGA, CGC, CGG, AGA, and AGG. Therefore it is appreciated that such substitutions in the coding region fall within the nucleic acid sequences covered by the present invention. Any and all of these sequences can be
15 utilized in the same way as described herein as a CBH catalytic domain or a cellulase catalytic domain.

Exemplary computer programs which can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, e.g., BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, publicly available on the
20 Internet at www.ncbi.nlm.nih.gov/BLAST/. See also, Altschul, *et al.*, 1990 and Altschul, *et al.*, 1997.

Sequence searches are typically carried out using the BLASTN program when evaluating a given nucleic acid sequence relative to nucleic acid sequences in the GenBank DNA Sequences and other public databases. The BLASTX program is
25 preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTN and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. (See, e.g., Altschul, *et al.*, 1997.)

30 A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is performed using for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

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In one exemplary approach, sequence extension of a nucleic acid encoding a CBH or EG catalytic domain may be carried out using conventional primer extension procedures as described in Sambrook *et al.*, *supra*, to detect CBH or bacterial EG precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA and/or to identify ORFs that encode the catalytic domain or full length protein.

In yet another aspect, the entire or partial nucleotide sequence of the nucleic acid sequence of the *T. reesei chb1* or GH5a-E1 may be used as a probe. Such a probe may be used to identify and clone out homologous nucleic acid sequences from related organisms.

Screening of a cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook *et al.*, (1989). Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook *et al.*, *supra*.

In addition, alignment of amino acid sequences to determine homology or identity between sequences is also preferably determined by using a "sequence comparison algorithm." Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), by visual inspection or MOE by Chemical Computing Group, Montreal Canada.

An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al.*, *J. Mol. Biol.* 215:403-410 (1990) and reference is also made to Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<www.ncbi.nlm.nih.gov>).

The heterologous cellulase fusion construct according to the invention may also include a terminator sequence. In some embodiments the terminator and the promoter are derived from the same source, for example a *Trichoderma* exo-cellobiohydrolase gene. In other embodiments the terminator and promoter are derived from different sources. In preferred embodiments the terminator is derived from a filamentous fungal source and particular a *Trichoderma*. Particularly suitable terminators include *cbh1*

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derived from a strain of *Trichoderma* specifically *T. reesei* and the glucoamylase terminator derived from *Aspergillus niger* or *A. awamori*. (Nunberg et al., 1984 and Boel et al., 1984).

The heterologous fusion construct or a vector comprising the fusion construct
5 may also include a selectable marker. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art. Typical selectable marker genes include *argB* from *A. nidulans* or *T. reesei*, *amdS* from *A. nidulans*, *pyr4* from *Neurospora crassa* or *T. reesei*, *pyrG* from *Aspergillus niger* or *A. nidulans*. Markers useful in vector systems for transformation of
10 *Trichoderma* are described in Finkelstein, Chap. 6, in BIOTECHNOLOGY OF FILAMENTOUS FUNGI, Finkelstein et al eds. Butterworth-Heinemann, Boston, MA 1992. The *amdS* gene from *Aspergillus nidulans* encodes the enzyme acetamidase that allows transformant cells to grow on acetamide as a nitrogen source (Kelley et al., EMBO J. 4:475-479 (1985) and Penttila et al., Gene 61:155-164 (1987)). The selectable marker
15 (e.g. *pyrG*) may restore the ability of an auxotrophic mutant strain to grow on a selective minimal medium and the selectable marker (e.g. *olic31*) may confer to transformants the ability to grow in the presence of an inhibitory drug or antibiotic

A typical heterologous cellulase fusion construct is depicted in Figures 1 and 14. Methods used to ligate a heterologous cellulase nucleic acid construct encompassed by
20 the invention and other heterologous nucleic acid sequences and to insert them into suitable vectors are well known in the art. Linking is generally accomplished by ligation at convenient restriction sites, and if such sites do not exist, synthetic oligonucleotide linkers are used in accordance with conventional practice. Additionally vectors can be constructed using known recombination techniques.

Any vector may be used as long as it is replicable and viable in the cells into
25 which it is introduced. Large numbers of suitable cloning and expression vectors are described in Sambrook et al., 1989, Ausubel FM et al., 1993, and Strathern et al., 1981, each of which is expressly incorporated by reference herein. Further appropriate expression vectors for fungi are described in van den Hondel, C.A.M.J.J. et al. (1991)
30 In: Bennett, J.W. and Lasure, L.L. (eds.) More Gene Manipulations in Fungi. Academic Press, pp. 396-428. The appropriate DNA sequence may be inserted into a vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by standard procedures. Such procedures and related sub-cloning procedures are deemed to be within the scope of knowledge of those
35 skilled in the art. Exemplary useful plasmids include pUC18, pBR322, pUC100,

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pSL1180 (Pharmacia Inc., Piscataway, NJ) and pFB6. Other general purpose vectors such as in *Aspergillus*, pRAX and in *Trichoderma*, pTEX maybe also be used (Figures 16 and 17).

5 B. Target Host Cells.

In one embodiment of the present invention, the filamentous fungal parent or host cell may be a cell of a species of, but not limited to, *Trichoderma* sp., *Penicillium* sp., *Humicola* sp., *Chrysosporium* sp., *Gliocladium* sp., *Aspergillus* sp., *Fusarium* sp., *Neurospora* sp., *Hypocrea* sp., and *Emericella* sp. As used herein, the term

10 "*Trichoderma*" or "*Trichoderma* sp." refers to any fungal strains which have previously been classified as *Trichoderma* or are currently classified as *Trichoderma*. Some preferred species for *Trichoderma* fungal parent cells include *Trichoderma longibrachiatum* (reesei), *Trichoderma viride*, *Trichoderma koningii*, and *Trichoderma harzianum* cells. Particularly preferred host cells include cells from strains of *T. reesei*,

15 such as RL-P37 (Sheir-Neiss, et al., *Appl. Microbiol. Biotechnol.* 20:46-53 (1984) and functionally equivalent and derivative strains, such as *Trichoderma reesei* strain RUT-C30 (ATCC No. 56765) and strain QM9414 (ATCC No. 26921). Also reference is made to ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56767 and NRRL 1509.

20 Some preferred species for *Aspergillus* fungal parent cells include *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus aculeatus*, and *Aspergillus nidulans* cells. In one embodiment, the strain comprises *Aspergillus niger*, for example *A. niger* var. *awamori* dgr246 (Goedegebuur et al, (2002) *Curr. Genet* 41: 89-98) and GCDAP3, GCDAP4 and GAP3-4 (Ward, M, et al., (1993), *Appl. Microbiol. Biotechnol.* 39:738-

25 743).

In some instances it is desired to obtain a filamentous host cell strain such as a *Trichoderma* host cell strain which has had one or more cellulase genes deleted prior to introduction of a heterologous cellulase fusion construct encompassed by the invention. Such strains may be prepared by the method disclosed in U.S. Patent No. 5,246,853,

30 U.S. Patent 5,861,271 and WO 92/06209, which disclosures are hereby incorporated by reference. By expressing a cellulase fusion protein or components thereof having cellulolytic activity in a host microorganism that is missing one or more cellulase genes, the identification and subsequent purification procedures are simplified. Any gene from *Trichoderma* sp. which has been cloned can be deleted, for example, the *cbh1*, *cbh2*,

35 *egl1*, and *egl2* genes as well as those encoding EG3 and/or EG5 protein (see e.g., U.S.

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Patent No. 5,475,101 and WO 94/28117, respectively). Gene deletion may be accomplished by inserting a form of the desired gene to be deleted or disrupted into a plasmid by methods known in the art.

Parental fungal cell lines are generally cultured under standard conditions with media containing physiological salts and nutrients, such as described by Pourquie, J. et al., BIOCHEMISTRY AND GENETICS OF CELLULOSE DEGRADATION, eds. Aubert J.P. et al., Academic Press pp. 71-86 (1988) and Ilmen, M. et al., *Appl. Environ. Microbiol.* 63:1298 -1306 (1997). Also reference is made to common commercially prepared media such as yeast Malt Extract (YM) broth, Luria Bertani (LB) broth and Sabouraud Dextrose (SD) broth.

C. Introduction of a Heterologous Cellulase Fusion Construct or Vector into Fungal Host Cells and Culture Conditions.

A host fungal cell may be genetically modified (*i.e.*, transduced, transformed or transfected) with a heterologous cellulase fusion construct according to the invention, a cloning vector or an expression vector comprising a heterologous cellulase fusion construct. The methods of transformation of the present invention may result in the stable integration of all or part of the construct or vector into the genome of the filamentous fungus. However, transformation resulting in the maintenance of a self-replicating extra-chromosomal transformation vector is also contemplated.

Many standard transformation methods can be used to produce a filamentous fungal cell line such as a *Trichoderma* or *Aspergillus* cell line that express large quantities of a heterologous protein. Some of the published methods for the introduction of DNA constructs into cellulase-producing strains of *Trichoderma* include Lorito, Hayes, DiPietro and Harman (1993) *Curr. Genet.* 24: 349-356; Goldman, VanMontagu and Herrera-Estrella (1990) *Curr. Genet.* 17:169-174; Penttila, Nevalainen, Ratto, Salminen and Knowles (1987) *Gene* 61: 155-164, EP-A-0 244 234 and also Hazell B. et al., 2000; for *Aspergillus* include Yelton, Hamer and Timberlake (1984) *Proc. Natl. Acad. Sci. USA* 81: 1470-1474; for *Fusarium* include Bajar, Podila and Kolattukudy, (1991) *Proc. Natl. Acad. Sci. USA* 88: 8202-8212; for *Streptomyces* include Hopwood et al., (1985) Genetic manipulation of streptomyces: a laboratory manual, The John Innes Foundation, Norwich, UK; and for *Bacillus* include Brigidi, DeRossi, Bertarini, Riccardi and Matteuzzi, (1990), *FEMS Microbiol. Lett.* 55: 135-138.

Other methods for introducing a heterologous cellulase fusion construct or vector into filamentous fungi (*e.g.*, *H. jecorina*) include, but are not limited to the use of

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a particle or gene gun (biolistics), permeabilization of filamentous fungi cells walls prior to the transformation process (e.g., by use of high concentrations of alkali, e.g., 0.05 M to 0.4 M CaCl_2 or lithium acetate), protoplast fusion, electroporation, or agrobacterium mediated transformation (US Patent 6,255,115).

5 An exemplary method for transformation of filamentous fungi by treatment of protoplasts or spheroplasts with polyethylene glycol and CaCl_2 is described in Campbell, et al., (1989) *Curr. Genet.* 16:53-56, 1989 and Penttila, M. et al., (1988) *Gene*, 63:11-22 and Penttila, M. et al., (1987) *Gene*, 61:155-164.

10 Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the heterologous gene.

 The invention includes the transformants of filamentous fungi especially *Trichoderma* cells comprising the coding sequences for the cellulase fusion protein.
15 The invention further includes the filamentous fungi transformants for use in producing fungal cellulase compositions, which include the cellulase fusion protein or components thereof.

 Following introduction of a heterologous cellulase fusion construct comprising the exoglucanase catalytic domain coding sequence and the endoglucanase catalytic
20 domain coding sequence, the genetically modified cells can be cultured in conventional nutrient media as described above for growth of target host cells and modified as appropriate for activating promoters and selecting transformants. The culture conditions, such as temperature, pH and the like, are those previously used for the host cell selected for expression, and will be apparent to those skilled in the art. Also
25 preferred culture conditions for a given filamentous fungus may be found in the scientific literature and/or from the source of the fungi such as the American Type Culture Collection (ATCC; "www.atcc.org").

 Stable transformants of filamentous fungi can generally be distinguished from unstable transformants by their faster growth rate and the formation of circular colonies
30 with a smooth rather than ragged outline on solid culture medium. Additionally, in some cases, a further test of stability can be made by growing the transformants on solid non-selective medium, harvesting the spores from this culture medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium.

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The progeny of cells into which such heterologous cellulase fusion constructs, or vectors including the same, have been introduced are generally considered to comprise the fusion protein encoded by the nucleic acid sequence found in the heterologous cellulase fusion construct.

5 In one exemplary application of the invention encompassed herein a recombinant strain of filamentous fungi, *e.g.*, *Trichoderma reesei*, comprising a heterologous cellulase fusion construct will produce not only a cellulase fusion protein but also will produce components of the cellulase fusion protein. In some embodiments the recombinant cells including the cellulase fusion construct will produce an increased
10 amount of cellulolytic activity compared to a corresponding recombinant filamentous fungi strain grown under essentially the same conditions but genetically modified to include separate heterologous nucleic acid constructs encoding an exo-cellobiohydrolase catalytic domain and/or a cellulase catalytic domain.

In some embodiments the cellulase fusion protein and components thereof
15 include the *Trichoderma reesei* CBH1 catalytic domain fused to the *Acidothermus cellulolyticus* E1 catalytic domain and the cleaved CBH1 and E1 products.

In some embodiments the cellulase fusion protein and components thereof include the *Trichoderma reesei* CBH1 catalytic domain fused to the *Acidothermus cellulolyticus* GH48 cellulase catalytic domain and the cleaved CBH1 and *Acidothermus*
20 *cellulolyticus* GH48 products.

In other embodiments the cellulase fusion protein and components thereof include the *T. reesei* CBH1 catalytic domain fused to a *Acidothermus cellulolyticus* GH74 endoglucanase catalytic domain and the cleaved CBH1 and GH74 products.

In other embodiments the cellulase fusion protein and components thereof
25 include the *T. reesei* CBH1 catalytic domain fused to a *Thermobifida fusca* E3 cellulase catalytic domain and the cleaved CBH1 and E3 products.

In other embodiments the cellulase fusion protein and components thereof include the *T. reesei* CBH1 catalytic domain fused to a *Thermobifida fusca* E5 endoglucanase catalytic domain and the cleaved CBH1 and E5 products.

30

D. Analysis of Protein Expression

In order to evaluate the expression of a cellulase fusion protein of the invention by a cell line that has been transformed with a heterologous cellulase fusion construct, assays can be carried out at the protein level, the RNA level or by use of functional

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bioassays particular to exo-cellobiohydrolase activity or endoglucanase activity and/or production.

In general, the following assays can be used to determine integration of cellulase fusion protein expression constructs and vector sequences, Northern blotting, dot blotting (DNA or RNA analysis), RT-PCR (reverse transcriptase polymerase chain reaction), *in situ* hybridization, using an appropriately labeled probe (based on the nucleic acid coding sequence), conventional Southern blotting and autoradiography.

In addition, the production and/or expression of a cellulase enzyme may be measured in a sample directly, for example, by assays for cellobiohydrolase or endoglucanase activity, expression and/or production. Such assays are described, for example, in Becker et al., Biochem J. (2001) 356:19-30; Mitsuishi et al., FEBS (1990) 275:135-138. Shoemaker et al. 1978; and Schulein 1988) each of which is expressly incorporated by reference herein. The ability of CBH1 to hydrolyze isolated soluble and insoluble substrates can be measured using assays described in Srisodsuk et al., J. Biotech. (1997) 57:49-57 and Nidetzky and Claeyssens Biotech. Bioeng. (1994) 44:961-966. Substrates useful for assaying exo-cellobiohydrolase, endoglucanase or β -glucosidase activities include crystalline cellulose, filter paper, phosphoric acid swollen cellulose, celooligosaccharides, methylumbelliferyl lactoside, methylumbelliferyl cellobioside, orthonitrophenyl lactoside, paranitrophenyl lactoside, orthonitrophenyl cellobioside, paranitrophenyl cellobioside.

In addition, protein expression, may be evaluated by immunological methods, such as immunohistochemical staining of cells, tissue sections or immunoassay of tissue culture medium, e.g., by Western blot or ELISA. Such immunoassays can be used to qualitatively and quantitatively evaluate expression of a cellulase, for example CBH. The details of such methods are known to those of skill in the art and many reagents for practicing such methods are commercially available.

In an embodiment of the invention, the cellulase fusion protein which is expressed by the recombinant host cell will be about 0.1 to 80% of the total expressed cellulase. In other embodiments, the amount of expressed fusion protein will be in the range of about 0.1mg to 100g; about 0.1mg to 50 g and 0.1 mg to 10g protein per liter of culture media.

E. Recovery And Purification Of Cellulase Fusion Proteins and Components Thereof.

In general, a cellulase fusion protein or components of the cellulase fusion protein produced in cell culture are secreted into the medium and may be recovered

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and optionally purified, e.g., by removing unwanted components from the cell culture medium. However, in some cases, a cellulase fusion protein or components thereof may be produced in a cellular form necessitating recovery from a cell lysate. In such cases the protein is purified from the cells in which it was produced using techniques
5 routinely employed by those of skill in the art. Examples include, but are not limited to, affinity chromatography (van Tilbeurgh *et al.*, FEBS Lett. 16:215, 1984), ion-exchange chromatographic methods (Goyal *et al.*, Bioresource Technol. 36:37-50, 1991; Fliess *et al.*, Eur. J. Appl. Microbiol. Biotechnol. 17:314-318, 1983; Bhikhabhai *et al.*, J. Appl. Biochem. 6:336-345, 1984; Ellouz *et al.*, J. Chromatography 396:307-317, 1987),
10 including ion-exchange using materials with high resolution power (Medve *et al.*, J. Chromatography A 808:153-165, 1998), hydrophobic interaction chromatography (Tomaz and Queiroz, J. Chromatography A 865:123-128, 1999), and two-phase partitioning (Brumbauer, *et al.*, Bioseparation 7:287-295, 1999).

Once expression of a given cellulase fusion protein or components thereof is
15 achieved, the proteins thereby produced may be purified from the cells or cell culture by methods known in the art and reference is made to Deutscher, Methods in Enzymology, vol. 182, no. 57, pp. 779, 1990; and Scopes, Methods Enzymol. 90: 479-91, 1982. Exemplary procedures suitable for such purification include the following: antibody-affinity column chromatography, ion exchange chromatography; ethanol precipitation;
20 reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, e.g., Sephadex G-75.

A purified form of a cellulase fusion protein or components thereof may be used to produce either monoclonal or polyclonal antibodies specific to the expressed protein
25 for use in various immunoassays. (See, e.g., Hu *et al.*, Mol Cell Biol. vol.11, no. 11, pp. 5792-5799, 1991). Exemplary assays include ELISA, competitive immunoassays, radioimmunoassays, Western blot, indirect immunofluorescent assays and the like.

F. Utility of enzymatic compositions comprising the cellulase fusion proteins or
30 components thereof.

The cellulase fusion protein and components comprising the catalytic domains of the cellulase fusion protein find utility in a wide variety applications, including use in detergent compositions, stonewashing compositions, in compositions for degrading wood pulp into sugars (e.g., for bio-ethanol production), and/or in feed
35 compositions. In some embodiments, the cellulase fusion protein or components

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thereof may be used as cell free extracts. In other embodiments the fungal cells expressing a heterologous cellulase fusion construct are grown under batch or continuous fermentation conditions. A classical batch fermentation is a closed system, wherein the composition of the medium is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells progress through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the production of products and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO₂. Batch and fed-batch fermentations are common and well known in the art.

Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen source is maintained at a fixed rate and all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems

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strive to maintain steady state growth conditions. Thus, cell loss due to medium being drawn off must be balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

In some applications, the cellulase fusion protein and components thereof find utility in detergent compositions, stonewashing compositions or in the treatment of fabrics to improve their feel and appearance. A detergent composition refers to a mixture which is intended for use in a wash medium for the laundering of soiled cellulose containing fabrics. A stonewashing composition refers to a formulation for use in stonewashing cellulose containing fabrics. Stonewashing compositions are used to modify cellulose containing fabrics prior to sale, *i.e.*, during the manufacturing process. In contrast, detergent compositions are intended for the cleaning of soiled garments and are not used during the manufacturing process.

In the context of the present invention, such compositions may also include, in addition to cellulases, surfactants, additional hydrolytic enzymes, builders, bleaching agents, bleach activators, bluing agents and fluorescent dyes, caking inhibitors, masking agents, cellulase activators, antioxidants, and solubilizers.

Surfactants may comprise anionic, cationic and nonionic surfactants such as those commonly found in detergents. Anionic surfactants include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; and alkanesulfonates. Ampholytic surfactants include quaternary ammonium salt sulfonates, and betaine-type ampholytic surfactants. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule. Nonionic surfactants may comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, fatty acid glycerine monoesters, and the like.

Cellulose containing fabric may be any sewn or unsewn fabrics, yarns or fibers made of cotton or non-cotton containing cellulose or cotton or non-cotton containing cellulose blends including natural cellulose and manmade cellulose (such as jute, flax, ramie, rayon, and lyocell). Cotton-containing fabrics are sewn or unsewn fabrics, yarns or fibers made of pure cotton or cotton blends including cotton woven fabrics, cotton knits, cotton denims, cotton yarns, raw cotton and the like.

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Preferably the cellulase compositions comprising the cellulase fusion protein or components thereof are employed from about 0.00005 weight percent to about 5 weight percent relative to the total detergent composition. More preferably, the cellulase compositions are employed from about 0.0002 weight percent to about 2 weight percent relative to the total detergent composition.

Since the rate of hydrolysis of cellulosic products may be increased by using a transformant having a heterologous cellulase fusion construct inserted into the genome, products that contain cellulose or heteroglycans can be degraded at a faster rate and to a greater extent. Products made from cellulose such as paper, cotton, cellulosic
diapers and the like can be degraded more efficiently in a landfill. Thus, the fermentation product obtainable from the transformants or the transformants alone may be used in compositions to help degrade by liquefaction a variety of cellulose products that add to the overcrowded landfills.

Cellulose-based feedstocks are comprised of agricultural wastes, grasses and woods and other low-value biomass such as municipal waste (e.g., recycled paper, yard clippings, etc.). Ethanol may be produced from the fermentation of any of these cellulosic feedstocks. However, the cellulose must first be converted to sugars before there can be conversion to ethanol. A composition containing an enhanced amount of cellulolytic activity due to the inclusion of a cellulase fusion protein or components thereof may find utility in ethanol production

Ethanol can be produced via saccharification and fermentation processes from cellulosic biomass such as trees, herbaceous plants, municipal solid waste and agricultural and forestry residues. However, the ratio of individual cellulase enzymes within a naturally occurring cellulase mixture produced by a microbe may not be the most efficient for rapid conversion of cellulose in biomass to glucose. It is known that endoglucanases act to produce new cellulose chain ends which themselves are substrates for the action of cellobiohydrolases and thereby improve the efficiency of hydrolysis of the entire cellulase system. Therefore, the use of increased or optimized endoglucanase activity from a cellulase fusion protein or components thereof may greatly enhance the production of ethanol and sugar which can be converted by fermentation to other chemicals.

Thus, the inventive cellulase fusion protein and components thereof find use in the hydrolysis of cellulose to its sugar components. In one embodiment, the cellulase fusion protein or components thereof are added to the biomass prior to the addition of a fermentative organism. In another embodiment, the cellulase fusion protein or

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components thereof are added to the biomass at the same time as a fermentative organism. Optionally, there may be other cellulase components present in either embodiment.

5

EXPERIMENTAL

The present invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention.

In the disclosure and experimental section, which follows, the following

10 abbreviations apply: CBH1-E1 (*T. reesei* CBH1 catalytic domain and linker fused to an *Acidothrmus cellulolyticus* GH5A endoglucanase I catalytic domain);

CBH1-48E (*T. reesei* CBH1 catalytic domain and linker fused to an *Acidothrmus cellulolyticus* GH48 cellulase catalytic domain);

CBH1-74E (*T. reesei* CBH1 catalytic domain and linker fused to an *Acidothrmus*

15 *cellulolyticus* GH74 endoglucanase catalytic domain);

CBH1-TfE3 (*T. reesei* CBH1 catalytic domain and linker fused to the CBD, linker and catalytic domain of *Thermobifida fusca* E3 cellulase; and

CBH1-TfE5 (*T. reesei* CBH1 catalytic domain and linker fused to the CBD, linker and catalytic domain of *Thermobifida fusca* E5 endoglucanase;

20 wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per minute); H₂O (water); dH₂O (deionized water); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); g (grams); µg (micrograms); mg (milligrams); µL (microliters); ml and mL (milliliters); mm (millimeters); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); MW (molecular weight); sec (seconds); min(s)

25 (minute/minutes); hr(s) (hour/hours); PAGE (polyacrylamide gel electrophoresis); phthalate buffer, (sodium phthalate in water, 20mN, pH 5.0); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); w/w (weight to weight); v/v (volume to volume); and Genencor (Genencor International, Inc., Palo

30 Alto, CA).

EXAMPLE 1

Construction of a CBH1-E1 Fusion Vector

The CBH1-E1 fusion construct included the *T. reesei cbhl* promoter; the *T.*

35 *reesei cbhl* gene sequence from the start codon to the end of the *cbhl* linker and an additional 12 bases of DNA 5' to the start of the endoglucanase coding sequence, a

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stop codon and the *T. reesei cbh1* terminator (see Figures 14 and 15). The additional 12 DNA bases (ACTAGTAAGCGG) (SEQ ID NO. 20) code for the restriction endonuclease *SpeI* and the amino acids Ser, Lys, and Arg.

The plasmid E1-pUC19 which contained the open reading frame for the E1 gene locus was used as the DNA template in a PCR reaction. Equivalent plasmids are described in USP 5,536,655 which also describes the cloning of the E1 gene from the actinomycete *Acidothermus cellulolyticus*, ATCC 43068, Mohagheghi A. et al., 1986. Standard procedures for working with plasmid DNA and amplification of DNA using PCT were employed (Sambrook et al., 1989 and 2001).

The following two primers were used to amplify the coding region of the catalytic domain of the E1 endoglucanase.

Forward Primer 1 = EL-316 (containing a *SpeI* site):

GCTTATACTAGTAAGCGCGCGGGCGGCGGCTATTGGCACAC (SEQ ID NO: 21)

Reverse Primer 2 = EL-317 (containing an *Ascl* site and **stop codon reverse complement**):

GCTTATGGCGCGCCTTAGACAGGATCGAAAATCGACGAC (SEQ ID NO: 22).

The reaction conditions were as follow using materials from the PLATINUM Pfx DNA Polymerase kit (Invitrogen, Carlsbad, CA): 1 µl dNTP Master Mix (final concentration 0.2mM); 1 µl primer 1 (final conc 0.5 µM); 1 µl primer 2 (final conc 0.5 µM); 2µl DNA template (final conc 50 - 200 ng); 1µl 50mM MgSO₄ (final conc 1mM); 5µl 10 x Pfx Amplification Buffer; 5µl 10 x PCRx Enhancer Solution; 1µl Platinum Pfx DNA Polymerase (2.5U total); 33µl water, for 50 µl total reaction volume.

Amplification parameters were: step 1 - 94°C for 2 min (1st cycle only to denature antibody bound polymerase); step 2 - 94°C for 45 sec; step 3 - 60°C for 30 sec; step 4 - 68°C for 2 min; step 5 - repeated step 2 for 24 cycles and step 6 - 68°C for 4 min.

The appropriately sized PCR product was cloned into the Zero Blunt TOPO vector and transformed into chemically competent Top10 *E. coli* cells (Invitrogen Corp., Carlsbad, CA) plated onto appropriate selection media (LA with 50 ppm kanamycin and grown overnight at 37°C. Several colonies were picked from the plate media and grown in 5 ml cultures at 37°C in selection media (LB with 50 ppm kanamycin) from which plasmid mini-preps were made.

Plasmid DNA from several clones was restriction digested to confirm the correct size insert. The correct sequence was confirmed by DNA sequencing. Following sequence verification, the E1 catalytic domain was excised from the TOPO vector by

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digesting with the restriction enzymes *SpeI* and *Ascl*. This fragment was ligated into the pTrex4 vector which had been digested with the restriction enzymes *SpeI* and *Ascl* (see Figures 16 and 17).

The ligation mixture was transformed into MM294 competent *E. coli* cells, plated onto appropriate selection media (LA with 50 ppm carbenicillin) and grown overnight at 37°C. Several colonies were picked from the plate media and grown overnight in 5 ml cultures at 37°C in selection media (LB with 50 ppm carbenicillin) from which plasmid mini-preps were made. Correctly ligated CBH1-E1 fusion vectors were confirmed by restriction digestion.

EXAMPLE 2

Transformation and Expression the CBH1-E1 Fusion Construct into a *T. reesei* host strain.

Various *T. reesei* strains were transformed with the CBH1-E1 fusion construct. The host strains included a derivative of *T. reesei* RL-P37 and a derivative of *T. reesei* wherein the native cellulase genes (*cbh1*, *cbh2*, *egl1*, and *egl2*) were deleted.

Approximately one-half swab (or 1 -2 cm²) of a plate of a sporulated *T. reesei* derivative of strain RL-P37 (Sheir-Neiss, *et al.*, 1984) mycelia (grown on a PDA plate for 7 days at 28°C) was inoculated into 50ml of YEG (5g/L yeast extract plus 20g/L glucose) broth in a 250 ml, 4-baffled shake flask and incubated at 30°C for 16-20 hours at 200 rpm.

The mycelia was recovered by transferring the liquid volume into 50ml conical tubes and spinning at 2500 rpm for 10 minutes. The supernatant was aspirated off. The mycelial pellet was transferred into a 250 ml, CA Corning bottle containing 40ml of B glucanase solution and incubated at 30°C, 200 rpm for 2 hrs to generate protoplasts for transformation. Protoplasts were harvested by filtration through sterile miracloth into a 50ml conical tube. They were pelleted by spinning at 2000 rpm for 5 minutes, and the supernatant was aspirated off. The protoplast pellet was washed once with 50ml of 1.2 M sorbitol, spun down, aspirated, and washed with 25ml of sorbitolCaCl₂. Protoplasts were counted and then pelleted again at 2000 rpm for 5 min, the supernatant was aspirated off, and the protoplast pellet was resuspended in a sufficient volume of sorbitol CaCl₂ to generate a protoplast concentration of 1.25 x 10⁸ protoplasts per ml constituting the protoplast solution.

Aliquots of up to 20 µg of expression vector DNA (in a volume no greater than 20 µl) were placed into 15ml conical tubes and the tubes were put on ice. Then 200µl of

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the protoplast solution was added, followed by 50 μ l PEG solution to each transformation aliquot. The tubes were mixed gently and incubated on ice for 20 min. Next an additional 2 ml of PEG solution was added to the transformation aliquot tubes, followed by gentle inversion and incubation at room temperature for 5 minutes. Next 4
5 ml of Sorbitol/ CaCl_2 solution was added to the tubes (generating a total volume of 6.2 ml). This transformation mixture was divided into 3 aliquots each containing about 2ml. An overlay mixture was created by adding each of these three aliquots to three tubes containing 10 ml of melted acetamide/sorbitol top agar (kept molten by holding at 50°C) and this overlay mixture was poured onto a selection plate of acetamide/sorbitol agar.
10 The transformation plates were then incubated at 30°C for four to seven days.

The transformation was performed with *amdS* selection. Acetamide/sorbitol plates and overlays were used for the transformation. For the selection plates, the same plates were used, but without sorbitol. Transformants were purified by transfer of isolated colonies to fresh selective media containing acetamide.

15 With reference to the examples the following solutions were made as follows.

1) 40 ml β -D-glucanase solution was made up in 1.2M sorbitol and included 600mg β -D-glucanase and 400mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Catalog No. 0439-1, InterSpex Products Inc., San Mateo, CA).

20 2) 200 ml PEG solution contained 50g polyethylene glycol 4000 (BDH Laboratory Supplies Poole, England) and 1.47g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ made up in dH_2O .

3) Sorbitol/ CaCl_2 contained 1.2M sorbitol and 50mM CaCl_2 .

4) Acetamide/sorbitol agar:

25 Part I - 0.6g acetamide (Aldrich, 99% sublime.), 1.68g CsCl, 20g glucose, 20g KH_2PO_4 , 0.6g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 ml 1000 x salts (see below), adjusted to pH 5.5, brought to volume (300 mls) with dH_2O , filtered and sterilized.

Part II - 20g Noble agar and 218g sorbitol brought to volume (700mls) with dH_2O and autoclaved.

30 Part II was added to part I for a final volume of 1L.

5) 1000 x Salts - 5g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ were combined and the volume was brought to 1L with dH_2O . The solution was filtered and sterilized.

35 6) Acetamide/sorbitol top agar is prepared as is acetamide/sorbitol agar except that top agar is substituted for noble agar.

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This procedure was similar to that described in Penttila et al., Gene 61: 155 – 164, 1987. Individual fungal transformants were grown up in shake flask culture to determine the level of fusion protein expression. The experiments were conducted essentially as described in example 1 of U. S. Patent 5,874,276 with the following modification: 16 g/L of alpha-lactose was substituted for cellulose in TSF medium. The highest level of cleaved E1 protein expression from a transformant in shake flasks was estimated to be greater than 3 g/L.

In general, the fermentation protocol as described in Foreman *et al.* (Foreman *et al.* (2003) *J. Biol. Chem* 278:31988-31997) was followed. Vogels minimal medium (Davis *et al.*, (1970) *Methods in Enzymology* 17A, pg 79 - 143 and Davis, Rowland, NEUROSPORA, CONTRIBUTIONS OF A MODEL ORGANISM, Oxford University Press, (2000)) containing 5% glucose was inoculated with 1.5 ml frozen spore suspension. After 48 hours, each culture was transferred to 6.2L of the same medium in a 14L Biolafitte fermenter. The fermenter was run at 25°C, 750 RPM and 8 standard liters per minute airflow. One hour after the initial glucose was exhausted, a 25% (w/w) lactose feed was started and fed in a carbon limiting fashion to prevent lactose accumulation. The concentrations of glucose and lactose were monitored using a glucose oxidase assay kit or a glucose hexokinase assay kit with beta-galactosidase added to cleave lactose, respectively (Instrumentation Laboratory Co., Lexington, MA). Samples were obtained at regular intervals to monitor the progress of the fermentation. Collected samples were spun in a 50ml centrifuge tube at 3/4 speed in an International Equipment Company (Needham Heights, MA) clinical centrifuge.

Shake flask grown supernatant samples were run on BIS-TRIS SDS -PAGE gels (Invitrogen), under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 18.

EXAMPLE 3

Assay of Cellulolytic Activity from Transformed *Trichoderma reesei* Clones

The following assays and substrates were used to determine the cellulolytic activity of the CBH1-E1 fusion protein.

Pretreated corn stover (PCS) – Corn stover was pretreated with 2% w/w H₂SO₄ as described in Schell, D. et al., *J. Appl. Biochem. Biotechnol.* 105:69 – 86 (2003) and followed by multiple washes with deionized water to obtain a pH of 4.5. Sodium acetate was added to make a final concentration of 50mM and this was titrated to pH 5.0.

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Measurement of Total Protein - Protein concentration was measured using the bicinchoninic acid method with bovine serum albumin as a standard (Smith P. K. et al., Biochem. 150:76-85, 1985).

5

Cellulose conversion (Soluble sugar determinations) was evaluated by HPLC according to the methods described in Baker et al., Appl. Biochem. Biotechnol. 70-72:395 – 403 (1998).

10 A standard cellulosic conversion assay was used in the experiments. In this assay enzyme and buffered substrate were placed in containers and incubated at a temperature over time. The reaction was quenched with enough 100 mM Glycine, pH 11.0 to bring the pH of the reaction mixture to at least pH10. Once the reaction was quenched, an aliquot of the reaction mixture was filtered through a 0.2 micron membrane to remove solids. The filtered solution was then assayed for soluble sugars
15 by HPLC as described above. The cellulose concentration in the reaction mixture was approximately 7%. The enzyme or enzyme mixtures were dosed anywhere from 1 to 60 mg of total protein per gram of cellulose.

In one set of experiments the percent conversion of 13.8% PCS (7.06% cellulose) at 55°C for 1 day was evaluated using 10 mg enzyme/g cellulose in 50 mM
20 acetate buffer at 55°C. Samples were agitated at 700 rpm. Comparisons were made between supernatants from growth of 1) a *T. reesei* parent strain which included the native cellulase genes and 2) a corresponding *T. reesei* CBH1-E1 fusion strain transformed according to the examples herein. Samples were quenched at various times up to 24 hours.

25 The results are presented in Figure 23 and it is observed that the CBH1-E1 fusion protein outperforms the parent. It took about 6 hours for the CBH1-E1 fusion protein to yield 20% cellulose conversion, while it requires 10 hours for the parent cellulase to reach 20% hydrolysis.

30

EXAMPLE 4

Transformation and Expression the CBH1-48E Fusion Construct into *T. reesei*

The CBH1-48E fusion construct was designed according to the procedures described below in example 1 with the following differences. The forward primer was designed to maintain the reading frame translation and include a Lys-Arg kexin

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cleavage site (underlined) at the end of the *cbh1* linker sequence. The reverse primer contained a stop codon at the end of the GH48E.

Primers were ordered with 5 prime phosphates to enable subsequent blunt cloning. The GH48 catalytic domain was amplified with the following forward and reverse primers:

GH48 forward primer bluntF4 –

CTAAGAGAAACGACCCGTACATCCAGCGGTTCTCACGATGTA (SEQ ID NO: 23)

GH48 reverse primer bluntR5 –

TTACCCGGATGGGAAGAGCATGCCAAAATCGGCGTTTCG (SEQ ID NO: 24).

Amplification was performed using Stratagene's Herculanase High Fidelity Polymerase (Stratagene, La Jolla, CA). An annealing temperature of 65°C was used. A DNA plasmid encompassing the GH48 catalytic domain was used as the template for PCR (approximately 0.2 ug of DNA). An equivalent method for isolating the GH48 gene is described in U.S. Pat. Appln. No. 2003/0096342.

The amplification reaction set up:

Component	in μ l
10X Herculanase Buffer	5
10 mM dNTPs	1.5
H ₂ O	39.5
Fwd primer (10 μ M)	1
Rev primer (10 μ M)	1
Template	1
Herculanase Polymerase (5U)	1
Total reaction volume	50

Cycling:

Segment	No. of cycles	Temp °C	hr:min:sec
1	1	95	00:03:00
2	10	95 annealing Temp 72	00:00:40 00:00:30 30 sec. per 500 bp
3	20	95 annealing Temp 72	00:00:40 00:00:30 30 sec per 500 bp + 10 sec/cycle
4	1	4	hold

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All PCR products were gel purified and treated with Mung Bean Nuclease to produce blunt ends prior to ligation. The amplified, blunted fragment was ligated into pTrex4 that had been digested with the restriction enzymes SpeI and AscI followed by nuclease digestion to remove the 3' overhangs and thereby creating blunt ends. The newly created vector was then transformed into *E. coli*. Plasmid DNA was isolated from the colonies of transformed *E. coli*. Since the amplified GH48 fragment could insert into pTrex4 in two different orientations, restriction digests were performed to discern clones with correctly oriented inserts. Putative clones were confirmed by DNA sequencing.

Transformation of the fusion vector was performed using biolistic transformation according to the teaching of Hazell, B. W. et al., Lett. Appl. Microbiol. 30:282-286 (2000).

Expression of the CBH1-48E fusion protein was determined as described above for expression of the CBH1-EI fusion protein in Example 3. The highest level of CBH1-GH48 protein expression from a transformant in shakes was estimated to be about 0.1 g/L.

Shake flask grown supernatant samples were run on BIS-TRIS SDS -PAGE gels (Invitrogen), under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 19.

EXAMPLE 5

Transformation and Expression the CBH1-74E Fusion Construct into *T. reesei*

The CBH1-74E fusion construct was designed according to the procedures described above in example 1 with the following differences. The forward primer was designed to maintain the reading frame translation and include a Lys-Arg kexin cleavage site (underlined). The reverse primer encodes a stop codon (the reverse complement - underlined) at the end of the catalytic domain.

Primers were ordered with 5 prime phosphates to enable subsequent blunt cloning. The GH74 catalytic domain was amplified with the following forward and reverse primers:

GH74 forward bluntF4 –

CTAAGAGAGCGACGACTCAGCCGTACACCTGGAGCAACGTGGC (SEQ ID NO: 25)

and

GH74 reverse bluntR4 – TTACGATCCGGACGGCGCACCAATGTCCCCGTATA (SEQ ID NO: 26).

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The amplification conditions and subsequent cloning are as described in Example 4, but with an annealing temperature of 60°C,

An isolated fragment of DNA encompassing the GH74 catalytic domain was used as the template for PCR (approximately 0.2 ug of DNA). U.S. Pat. Appl. No. 2003/0108988 describes the cloning of GH74. (GH74 is referred to as AvIII in the published patent application.

After amplification, the amplified fragment was blunt end ligated into the pTrex4 vector that had been digested with *SpeI* and *AscI* followed by nuclease digestion to remove the 3' overhang creating blunt ends. The subsequent vector with the fusion construct was confirmed by sequencing.

Transformation of the fusion vector into *T. reesei* was performed using biolistic transformation according to Hazell B. et al., Lett. Appl. Microbiol. 30:282 - 286 (2000).

Expression of the CBH1-74E fusion protein was determined as described above for expression of the CBH1-E1 fusion protein in Example 2. The highest level of cleaved GH74 protein expression from a transformant in shake flasks was estimated to be greater than 3 g/L.

Shake flask grown supernatant samples were run on BIS-TRIS SDS -PAGE gels (Invitrogen), under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 20.

EXAMPLE 6

Transformation and Expression the CBH1-TfE3 Fusion Construct into *T. reesei*

The CBH1-TfE3 fusion construct was designed according to the procedures described above in Example 1 with the following differences.

The following primers were used to amplify the TfE3 cellulase - EL-310 forward (which contains a *SpeI* site)

GCTTATACTAGTAAAGCGCGCCGGCTGCTCGGTGGACTACACG (SEQ ID NO: 27) and EL-311 reverse (which contains a *AscI* site) –

GCTTATGGCGCGCCTTACAGAGGCGGGTAGGCGTTGG (SEQ ID NO: 28).

The plasmid containing the TfE3 gene was used as the DNA template for amplification. An equivalent template DNA is described in Zhang, S. et al., Biochem. 34:3386 - 3395, 1995. The amplification conditions and subsequent cloning are as described in example 1. Vector construction and biolistic transformation of *T. reesei* proceeded as described above.

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The highest level of expression in shake flasks was estimated to be greater than 0.4 g/L.

Shake flask grown supernatant samples were run on BIS-TRIS SDS -PAGE gels (Invitrogen), under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 21.

EXAMPLE 7

Transformation and Expression the CBH1-TfE5 Fusion Construct into *T. reesei*

The CBH1-TfE5 fusion construct was designed according to the procedures described above in Example 1 with the following differences. A plasmid equivalent to that described in Collmer & Wilson, Bio/Technol. 1:594 - 601 (1983) carrying the TfE5 gene was used as the DNA template to amplify the TfE5.

The following primers were used to amplify the TfE5 endoglucanase. EL-308 (which contains a SpeI site) – forward primer

GCTTATACTAGTAAAGCGCGCCGGTCTCACCGCCACAGTCACC (SEQ ID NO: 29)

and EL-309 (which contains a AscI site) reverse primer –

GCTTATGGCGCGCCTCAGGACTGGAGCTTGCTCCGC (SEQ ID NO: 30).

Transformation was as described above. The highest level of cleaved TfE5 protein expression from a transformant in shake flasks was estimated to be greater than 2 g/L.

Shake flask grown supernatant samples were run on BIS-TRIS SDS -PAGE gels (Invitrogen), under reducing conditions with MOPS (morpholinepropanesulfonic acid) SDS running buffer and LDS sample buffer. The results are provided in Figure 22.

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